APPLICATION OF ADENOVIRUS EXPRESSION SYSTEM FOR THE PRODUCTION OF RECOMBINANT CHOLINESTERASES

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ABSTRACT

Previous studies in rodents and nonhuman primates demonstrated that pretreatment of animals with cholinesterases (ChEs) could provide significant protection against behavioral and lethal effects of nerve agent intoxication. Currently, human serum butyrylcholinesterase (BChE) is under development as medical countermeasure against organophosphate chemical warfare agent toxicity. In this study, we report the expression of human BChE and bovine acetylcholinesterase (AChE) using the newly designed adenovirus expression system. The shuttle vector employed in this expression system contains cytomegalovirus-5 (CMV-5) promoter that was 5 to 10 times more efficient than the original CMV-1 promoter in inducing transgene expression in human embryonic epithelial cell line, namely 293 A. Both BChE and AChE were fully expressed as tetrameric forms by the simultaneous expression of proline-rich attachment domain in 293 A cells. By optimizing the culture conditions, 12-15 U/ml of AChE and 1.5-2.0 U/ml of BChE were produced with this system. The expressed rHu BChE was purified to homogeneity by a two-step procedure involving procainamide affinity column and nickel affinity column. The physico-chemical properties of BChE were found to be similar to those of the native protein. In addition, purified recombinant BChE showed catalytic properties similar to those of the native protein. These studies suggest that it is possible to produce milligram to gram quantities of homogeneous preparations of recombinant tetrameric AChE and BChE in tissue culture using the adenovirus expression system.

INTRODUCTION

Organophosphorous (OP) compounds are produced worldwide as chemical weapons and are a great threat to our soldiers. They produce toxicity by inactivating acetylcholinesterase (AChE) the enzyme responsible for the breakdown of the neurotransmitter, acetylcholine. The resultant increase in the level of acetylcholine at cholinergic synapses, particularly in the brain and diaphragm produces an acute cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation, behavioral incapacitation, muscular weakness, and convulsions culminating in death by respiratory failure. Current antidotal regimen for OP poisoning includes a combination of pretreatment with a spontaneously reactivating AChE inhibitor such as pyridostigmine bromide,

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postexposure therapy with anticholinergic drugs such as atropine sulfate, and oximes such as 2-PAM chloride. These antidotal regimens were successful in preventing lethality of animals to OP poisoning but were unable to prevent postexposure incapacitation, convulsions, performance deficits, or in many cases, permanent brain damage. These problems prompted the development of alternate protective measures capable of providing protection against the lethality of OP compounds as well as preventing postexposure incapacitation.

One approach is the use of enzymes, such as cholinesterases (ChEs), as bioscavengers of highly toxic OPs. ChEs are closely related serine hydrolases that can irreversibly bind and inactivate OP toxic agents before they reach physiological targets. ChEs are made of four identical subunits (574 amino acids/BChE subunit and 583 amino acids/AChE subunit), each containing one active site (2). Exogenously administered plasma-derived butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) protected animals from a variety of multiple LD_{50}'s of highly toxic OPs without any toxic effects or performance decrements (3-6). Based on data from animal experiments, it has been estimated that a dose of 200 mg of human serum (Hu) BChE would be required to provide protection against 2 x LD_{50} of soman without the need for immediate postexposure therapy (7). But the purification and production of native ChEs in such quantities from physiological sources is tedious, cumbersome, expensive, and requires large quantities of plasma. In this study, we explored whether the adenovirus expression system (commercially available as AdenoVATOR vector system) that is designed for high expression levels of functional proteins *in vitro* and *in vivo*, is suitable for the production of ChE. Results suggest that high-expression levels of tetrameric ChEs is possible with the AdenoVATOR vector expression system and that the system can be scaled up to produce milligram to gram quantities of these enzymes by growing 293 host cells in suspension cultures.

METHODS

Recombinant adenoviruses: Recombinant adenoviruses expressing Hu BChE (Ad-BChE), Bovine AChE (Ad-AChE) and proline-rich attachment domain (Ad-PRAD) were generated as described in the applications manual (Qbiogene Inc, Carlsbad, CA).

Cell culture and infections with recombinant adenoviruses: 293A cells (5 x 10^4) were seeded in 6-well plates. After 24 h, the cells were infected at different amounts of 4^{th} cycle CVE of Ad-BChE or Ad-BChE alone or in combination with Ad-PRAD for 1 h using 500 µl of infection medium (DMEM containing 2 % FBS, antibiotics penicillin and streptomycin, and sodium pyruvate) at 37^{o}C in the incubator. To the infected cells, 1.5 ml of growth medium (DMEM containing 10 % FBS, 50 µg/ml penicillin and streptomycin, and 50 µg/ml sodium pyruvate) was added and they were returned to the incubator. BChE and AChE activities in the culture medium were quantified according to Ellman et al (8) using 5,5'-dithiobis(2-nitrobenzoic acid in 50 mM sodium phosphate buffer pH 8.0 with butyrylthiocholine and acetylthiocholine as substrates, respectively.

In some experiments, ultra culture medium (Life Technologies Inc., Gaithersburg, MD) was used for infection of 293 A cells as described above as well as for the growth of infected cells. BChE and AChE activities were monitored daily in the culture medium. The culture media from 4 days post-infected cells was concentrated using YM-30 Amicon concentrators and used Western blotting and sucrose gradient centrifugation analyses.

Sucrose gradient centrifugation: Aliquots of recombinant ChEs (approximately 1 to 2 units) were mixed with catalase (11.3S, used as a sedimentation marker) and applied to linear 5-20%

sucrose gradients prepared in 50 mM sodium phosphate, pH 8.0. The gradients were centrifuged at 75,000 X g for 18 h at 4°C in an SW41Ti rotor (Beckman Instruments, Fullerton, CA). Gradients were fractionated from the top using an AutoDensiflow IIC (Buchler Instruments, Lenexa, KS), and fractions were assayed for AChE or BChE using the micro-Ellman assay (9).

Recombinant Hu BChE expression and purification: 293A cells (10 x 10^6) were seeded in 150 cm^2 tissue culture dishes. After 24 h, the cells were simultaneously infected with 10 µl of 4th cycle CVE of Ad-BChE and 5 µl of Ad-PRAD for 1 h using 10 ml of infection medium (DMEM containing 2 % FBS, antibiotics penicillin and streptomycin, and sodium pyruvate) at 37°C in the incubator. To the infected cells, 15 ml of growth medium (DMEM containing 10 % FBS, 50 µg/ml penicillin and streptomycin, and 50 µg/ml sodium pyruvate) was added and they were returned to the incubator for 7 to 8 days. Fifty to seventy culture dishes were infected in a single experiment. When BChE activity in the culture medium reached between 1.5 to 2.0 U/ ml, it was collected and subjected to centrifugation at 2500 rpm for 15 min at 4°C to remove cells and debris. The clear culture medium was used for the purification of recombinant Hu BChE by affinity chromatography using procainamide-Sepharose and nickel-affinity resin.

Thermal stability. Aliquots of rBChE at a concentration of 1 U/ml in 50 mM sodium phosphate, pH 8.0 containing 0.5% BSA were incubated at different temperatures (25-55°C). Samples were monitored for BChE activity after 30 min, 1 h, and 3 h using the Ellman assay (8).

Other methods. SDS-Polyacrylamide gel electrophoresis and Western blotting were performed as described elsewhere (9,10). Determination of kinetic constants (*Km*, *Kss*, *kcat*) and inhibition constants (*Ki*, α*Ki*) were performed as described (8,12). Protein concentration was determined using BCA protein assay kit (Pierce co, Rockford, IL) using the enhanced method according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Expression of ChEs in Ad-ChEs infected 293 cells: We examined the expression levels of recombinant (r) Hu BChE and rBo AChE in 293 cells infected with 0.5 µl of 4th cycle crude extract of Ad-BChE and Ad-AChE, respectively. Ad-ChEs infected cells were grown either in ultra culture medium or in DMEM containing 10% fetal bovine serum for 7 days and monitored for ChE activity (8) on selected days. As shown in Figures 1A and 1B, expression levels of rChEs were 5 to 6-fold higher in Ad-ChE infected cells when they were cultured in growth medium containing 10% FBS compared to ultra culture medium.

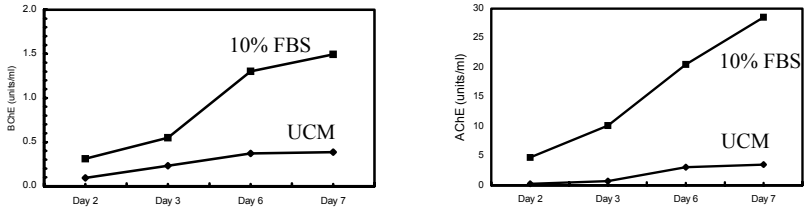

Figure 1. Time course for the expression of r Hu BChE and rBo AChE in 293 A cells.

Molecular forms of rChEs: To determine the composition of the various forms of rChEs produced in ultrapure culture medium, we performed sucrose gradient centrifugation analysis. As shown in Figure 2B, rHu BChE is made of mostly monomers and dimers (85%) and very little of tetramers (15%). In contrast, co-expression of PRAD resulted in the formation of mostly tetrameric rHu BChE (95%) (Figure 2C). Similarly, without PRAD, rBo AChE was monomeric (Figure 2E) but with PRAD co-expression, it consists of a mixture of monomers and dimers

(40%) and tetramers (60%) (Figure 2F). Human serum BChE (Figure 2A) and FBS AChE (Figure 2D) were included as positive controls. Collectively, these data suggest that the rChEs produced by the adenovirus expression system are mostly tetrameric in nature.

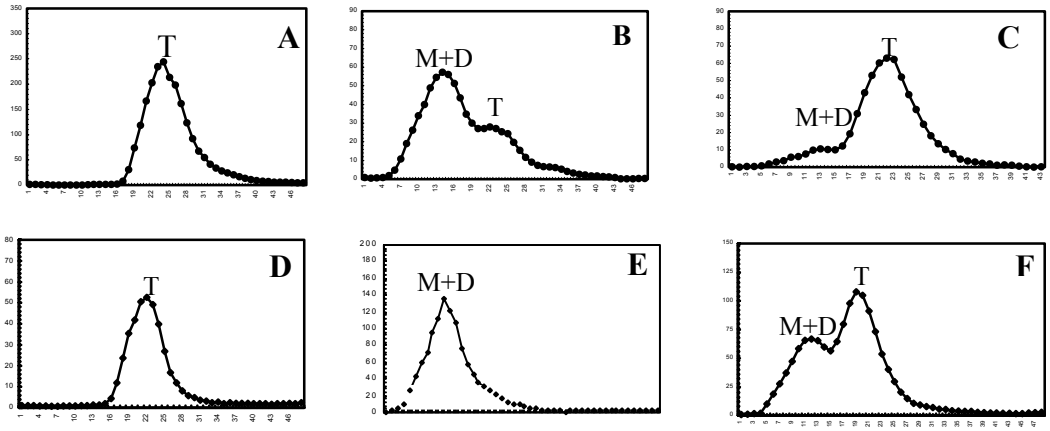

Figure 2: Sucrose gradient sedimentation analysis of rChEs Panels (A-F) represent the following: A, Hu BChE; B, rHu BChE made in ultra culture medium without PRAD; C, rHu BChE made in ultra culture medium with PRAD co-expression; D, native FBS AChE; E, rBo AChE made in ultra culture medium without PRAD; and F, rBo AChE made in ultra culture medium with PRAD co-expression. M+D, monomer+dimmer; and T, Tetramer. In all the panels, X axis, O.D at 412 nm and Y axis, fraction number.

We also tested whether the molecular composition of rHu BChE produced in growth medium containing DMEM and 10% FBS is different from that of the rHu BChE produced in ultra culture medium. 60 to 70% of the rHu BChE produced by 293 cells grown in serum containing medium is tetrameric as opposed to 15 to 20% for the rHu BChE produced in ultrapure culture medium (data not shown). These studies suggest that FBS contains factor(s) that promote the association of BChE monomers into tetramers.

Physico-chemical properties of rHu BChE. We compared the electrophoretic mobility of purified rHu BChE to that of the native BChE in polyacrylamide gels by subjecting the proteins to reducing/denaturing, non-reducing/denaturing, and non-reducing/non-denaturing conditions. As shown in Figures 3A, 3B, and 3C the mobility patterns of reduced/denatured rHu BChE, non-reduced/denatured rHu BChE and non-reduced/non-denatured rHu BChE were similar to those of the native BChE. These results suggest that rHu BChE made by the adenovirus expression system is structurally intact. Likewise, by western blotting analysis using a monoclonal antibody against FBS AChE, we found that the rBo AChE produced by the adenovirus expression system is a full-length molecule with a molecular size of 70 kDa (Figure 3D).

To determine whether rHu BChE produced by the adenovirus expression system is glycosylated, we treated both native and rHu BChEs with peptide N-glycosidase F and compared their elctrophoretic mobility on polyacrylamide gels. As shown in Figure 4, treatment of rHu BChE with peptide N-glycosidase F led to a shift in the mobility of the enzyme that is identical to

the native enzyme. These observations suggest that the rHu BChE produced by the adenovirus expression system is fully glycosylated.

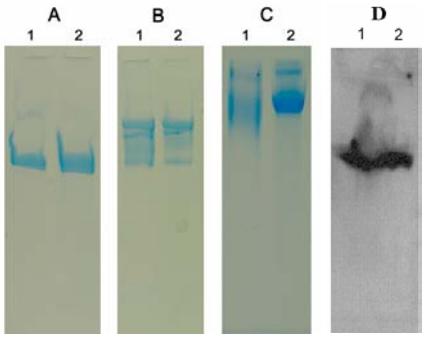

Figure 3: Polyacrylamide gel analysis of recombinant and native ChEs: Purified recombinant and native BChEs (5 μg) were analyzed using 4 to 15% gradient or 6 % polyacrylamide gels under reducing/denaturing (A), non-reducing/denaturing (B), or non-reducing/non-denaturing conditions (C). In all the panels, lane 1, rHu BChE, and lane 2, native Hu BChE. Recombinant Bo AChE (0.25 μg) was identified by SDS-PAGE followed by Western blotting (D). In panel D, lane 1, native FBS AChE and lane 2, rBo AChE.

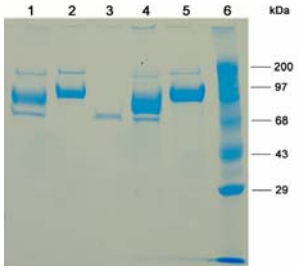

Figure 4. Glycosylation of rHu BChE. Glycosylation of rHu BChE was assessed by its sensitivity to peptide N-glycosidase F. Native and recombinant Hu BChEs (5 μg) were incubated at 30°C overnight with peptide N-glycosidase F in 50 mM phosphate buffer pH 8.0. Substrate to enzyme ratio was maintained at 10:1. Following incubation, samples were reduced and heat denatured and assayed by SDS-PAGE using 4 to 15 % gradient polyacrylamide gels. Gel lanes are as follows: lanes 2 and 5, recombinant and native Hu BChE respectively; lanes 1 and 4, recombinant and native Hu BChE after incubation with peptide N-glycosidase F; and lane 6, molecular weight markers.

We performed detailed kinetic studies and found that *Km*, *Kss*, and *kcat* for the rHu BChE were not significantly different from those of the native Hu BChE (Table 1). Likewise, the inhibition constants for the classical active-site inhibitor ethopropazine were similar to those of the native enzyme (data not shown).

TABLE 1. Catalytic properties of rHu BChE.

	Kinetic parameter		
Hu BChE	*Km* (μM)	*Kss* (mM)	*Kcat* (min^{-1})
Native	12.4 ± 3	0.78 ± 0.12	33990 ± 15078
Recombinant	13.0 ± 3	0.79 ± 0.11	32925 ± 11321

Values are means ± S.D for three independent experiments.

Thermal stability of rHu BChE was compared with that of the native Hu BChE. As shown in Table 2, recombinant and native Hu BChEs were thermally stable at temperatures up to

45°C. However, at temperatures above 51°C, it was thermally less stable than the native Hu BChE.

TABLE 2. Thermal stability of rHu BChE[a]

Temp (°C)	% Activity					
	30 min		60 min		180 min	
	Hu BChE	rHu BChE	Hu BChE	rHu BChE	Hu BChE	rHu BChE
25	100	100	100	100	100	100
35	100	100	100	100	100	99
45	100	99	100	96	100	95
51	83	80	80	48	68	30
55	46	16	36	7	0	0

a: Aliquots of enzyme containing rHu BChE and native Hu BChE at a concentration of 1U/ml in 50 mM sodium phosphate, pH 8.0, 0.5% BSA were incubated at indicated temperatures. BChE activity remaining after 30 min, 60 min, and 180 min of incubation was assayed according to Ellman et al (8).

CONCLUSIONS

We constructed and characterized recombinant adenoviruses expressing Hu BChE (Ad-BChE), bovine AChE (Ad-AChE) and PRAD (Ad-PRAD). Our studies suggest that the expression of BChE and AChE was 5 to 6 fold greater when the Ad-ChE infected cells were cultured in DMEM containing 10% FBS as compared to ultra culture medium. Sucrose gradient centrifugation analysis revealed that the recombinant ChEs produced in the ultra culture medium were made of mostly monomers and dimers. Co-expression of PRAD resulted in the conversion of monomeric ChEs into tetraemric ChEs. SDS-PAGE followed by western blotting revealed that the subunit molecular size of recombinant AChE was very similar to that of native AChE. Recombinant Hu BChE was purified to homogeneity using procainamide-Sepharose and metal-affinity resin chromatographies. Approximately 2 mg of purified rHu BChE was produced from 1.5 liters of culture media. Sucrose gradient centrifugation analysis revealed that the rHu BChE was mostly tetrameric. Analysis of the rHu BChE by PAGE under native and denaturing conditions revealed that it is structurally intact. The physico-chemical properties of rHu BChE were compared with those of native Hu BChE and found to be similar. Recombinant Hu BChE produced by CHO cells stably transfected with Hu BChE cDNA consisted of a mixture of monomers, dimers, and tetramers (13). Using the adenovirus expression system described

here, it is possible to produce mostly tetrameric rHu BChE with physico-chemical properties very similar to those of the native Hu BChE.

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